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Sequence-equivalent bacterial ASVs occupy dynamic network roles across amphibian development

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Research Article

Keywords: Community assembly, Core microbiota, Host filtering, Skin microbiota, SPIEC-EASI, Rhinella spinulosa.

Posted Date: October 13th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11185455/v1

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Additional Declarations: No competing interests reported.

Abstract

Host-associated microbiotas arise from interactions between environmental microbial communities and host-associated ecological filtering. However, how their organization changes during host development while specific bacterial lineages persist remains poorly understood. Here, we characterized the skin microbiota of pre-metamorphic *Rhinella spinulosa* tadpoles and compared it with the surrounding aquatic microbial pool, integrating these data with previously published juvenile and adult datasets reprocessed under a common analytical framework.

Tadpole skin represented a structured subset of the sampled aquatic microbial pool, containing both environmentally shared and skin-restricted ASVs. Core ASVs showed higher betweenness and were disproportionately represented among degree-defined hubs, linking consistent host association with network organization. Network architecture differed across developmental stages: connectivity, clustering, heterogeneity, and centralization peaked in juveniles, whereas the adult network contracted and fragmented. Sequence-level matching identified 44 ASVs detected across all three stages. These sequence-equivalent ASVs were more abundant and, in the primary analysis, those detected through adulthood showed lower degree but higher betweenness. Sensitivity analyses provided partial support for the betweenness pattern but not the degree pattern.

Most ASVs detected across all three stages remained non-hubs, while others gained or lost hub status across development. Thus, persistence of bacterial identity across stages did not imply persistence of network role. Our findings indicate that host filtering, stage-associated network reorganization, and lineage persistence are complementary dimensions of amphibian skin microbiota assembly, with taxonomic continuity coexisting with substantial changes in community structure.

Introduction

Host-associated microbiotas are integral to animal biology, contributing to nutrition, immune function, pathogen resistance, disease resilience, and host development [1–2]. These communities are structured ecological systems shaped by microbial interactions, host-associated filters, and environmental sources, and assembled through dispersal, selection, diversification, and ecological drift [3–4].

Most microbiota studies focus on composition and diversity [5], but ecological importance cannot always be inferred from abundance alone. Community organization may emerge from associations among taxa [6] that reorganize with host physiology and environmental conditions. Network approaches identify modules, highly connected taxa, and putative ecological associations [7–10].

The core microbiota comprises microorganisms consistently associated with a host across individuals or populations [11]. Core taxa are often assumed to be ecologically important because their prevalence indicates consistent association with host conditions [12–13]. However, consistent occurrence does not necessarily imply ecological importance, and few studies have examined whether core taxa occupy prominent network positions. Repeated detection of bacterial lineages across host development may

expose microbial lineages to changes in host physiology, habitat, and community composition. Linking prevalence and ontogenetic persistence with network topology may therefore clarify how host-associated communities are assembled and reorganized through time.

Amphibians undergo dramatic ontogenetic transitions involving changes in habitat, physiology, immune function, and skin structure [14], potentially modifying microbial composition and associations. Whether bacterial lineages detected before metamorphosis remain detectable through these transitions, and whether their network positions are conserved or reorganized, remains poorly understood. Previous studies show that amphibian skin microbiotas vary across host species, developmental stages, and environmental conditions: host ecology explained more variation than phylogeny among Malagasy frogs [15]; distinct communities occurred between tadpoles and postmetamorphic *Rana cascadae* [16]; and selection, persistence, and immigration have been described in salamanders [17]. Thus, amphibian skin provides a framework for investigating microbial reorganization over time.

Rhinella spinulosa is a widely distributed Andean amphibian inhabiting diverse environments across western South America. Previous work demonstrated marked environmental adaptation across populations [18] and characterized juvenile and adult skin-associated microbiotas [19]. These studies provide an opportunity to investigate how host filtering, network reorganization, and persistence interact during development, including whether retained bacterial lineages occupy distinctive topological positions or change network roles.

Here, we characterized the larval skin-associated microbiota of *R. spinulosa* and integrated these data with previously published juvenile and adult datasets reprocessed under a common analytical framework. We characterized partitioning between tadpole skin and the surrounding aquatic water-column microbial pool, identified the prevalence-defined core microbiota, compared network architecture across developmental stages, and tracked bacterial lineages detected across all stages to examine how persistence relates to network topology. We hypothesized that (i) host-associated filtering would generate structured partitioning between tadpole skin and the sampled aquatic microbial pool, resulting in distinct bacterial occurrence, prevalence, and abundance; (ii) developmental stage would be associated with extensive network reorganization; and (iii) a subset of bacterial lineages established during the pre-metamorphic stage would persist across development and exhibit distinctive topological positions within stage-specific networks. By integrating taxonomic and network approaches, this study provides new insights into the ecological organization of amphibian skin microbial communities.

Methods

Sample collection

All animal procedures were approved by the corresponding institutional ethics committees. Tadpole and aquatic water-column sampling were conducted in Farellones (2,460 m a.s.l.), Central Andes, Región Metropolitana, Chile (33°20'59"S, 70°18'34"W). Three water-column samples (1 L each; 14.8°C; pH 7.5)

were collected from the breeding pond and filtered through sterile 0.22 μm membrane filters (Merck). Sterile PBS and unused filters served as negative controls; no DNA was recovered, and they yielded no sequencing libraries. DNA was extracted directly from the filters. Five pre-metamorphic *Rhinella spinulosa* tadpoles (Gosner stage ≤ 25) [20] were captured, anesthetized with tricaine methanesulfonate (MS-222; Merck), and their dorsal surfaces swabbed with sterile cotton swabs. Individuals were released immediately, and swabs were preserved in sterile PBS until DNA extraction using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research).

16S rRNA gene sequencing and sequence processing

DNA libraries were prepared and sequenced by Macrogen Inc. (Seoul, Republic of Korea), targeting the V3–V4 region of the bacterial 16S rRNA gene on an Illumina MiSeq platform (2 × 300 bp paired-end). Raw sequences generated here were deposited in the Sequence Read Archive (PRJNA1529848). Publicly available skin microbiota datasets from juvenile (n = 10) and adult (n = 4) R. spinulosa from the Central Andes (Yerba Loca and La Parva, respectively) were retrieved from SRA (PRJNA1003691) [19]. Because each developmental stage was represented by a different locality, developmental stage and locality were fully confounded in the ontogenetic comparisons.

To ensure methodological consistency, tadpole, juvenile, and adult sequence data were reprocessed using the same QIIME 2 workflow (qiime2-amplicon-2024.10) [21]. Paired-end reads were imported and primers removed using Cutadapt [22] (error rate 0.1; minimum overlap 3 bp; untrimmed reads discarded), followed by quality filtering and denoising with Deblur (denoise-16S) [23]. Sequences were truncated to 199 bp (trim-length = 199; left-trim-len = 0), generating ASVs under a common framework. Taxonomy was assigned with classify-sklearn in the QIIME 2 feature-classifier plugin against SILVA SSU release 138 [24]. Downstream processing was performed in Phyloseq [25]. Only bacterial ASVs were retained; mitochondria, chloroplast sequences, and ASVs with zero abundance after filtering were removed.

Host filtering

ASVs were classified according to occurrence in pre-metamorphic tadpole skin and the sampled aquatic water-column microbial pool as skin-restricted, water-restricted, or shared. Skin prevalence was the number of tadpoles in which each ASV was detected, and the core skin microbiota comprised ASVs occurring in ≥ 80% of individuals (≥ 4 of 5 tadpoles).

Combining water occurrence with core membership, ASVs were assigned to five mutually exclusive categories: water only, shared (non-core), skin only (non-core), core (shared), and core (skin-restricted). Mean relative abundance was calculated separately for skin and water after converting ASV counts to sample-wise proportions. Taxonomic composition was summarized at the genus level after excluding unclassified and uncultured assignments.

To assess sensitivity to low-abundance water detections, ecological classification was repeated using water-column relative-abundance thresholds of > 0%, 0.01%, or 0.1% in at least one sample, while skin

occurrence and core membership remained fixed. ASVs below each threshold were reclassified as skin-restricted. The presence/absence criterion (> 0%) was retained for primary analyses because it provided the most conservative definition of water-column sharing.

Because environmental sampling was restricted to the water column, water-restricted and skin-restricted classifications were interpreted relative to the sampled aquatic microbial pool and did not exclude unsampled sources such as sediments or biofilms.

Differences in mean relative abundance and skin prevalence among the four skin-associated categories were evaluated using Kruskal-Wallis tests followed by Dunn's post hoc comparisons with Benjamini-Hochberg correction. Associations between skin prevalence and mean relative abundance were assessed using Spearman's rank correlation.

Differential abundance between tadpole skin and water was evaluated at the ASV level using ANCOM-BC2 [26], with environment (skin or water) as a fixed effect, Benjamini-Hochberg correction, and $\alpha = 0.05$. No additional prevalence or library-size filtering was applied (prv_cut = 0, lib_cut = 0); structural-zero detection and negative lower-bound correction were enabled. Among environmentally shared ASVs tested by ANCOM-BC2, core membership and differential-abundance status were compared using Pearson's χ^2 test, with Cramer's V as effect size.

Network inference

Microbial association networks were inferred separately for the complete tadpole skin microbiota and its prevalence-defined core fraction. The complete network included all 875 ASVs detected across five skin samples, whereas the core network included the 537 ASVs occurring in ≥ 80% of tadpoles. Networks were inferred using SPIEC-EASI in SpiecEasi v2.0.0 [27], with Meinshausen-Bühlmann neighborhood selection (method = "mb"), lambda.min.ratio = 1×10^{-2}, nlambda = 20, and 50 pulsar replicates (rep.num = 50).

Adjacency matrices were converted to graph objects using igraph and exported to Cytoscape v3.10.4 [28] via RCy3 [29] for visualization and topological analyses. Tadpole, juvenile, and adult networks were independently inferred using the same analytical framework.

Network topology

Network topology was characterized in Cytoscape v3.10.4 using Network Analyzer. Node degree and betweenness centrality [30] were extracted to characterize connectivity and bridging position, respectively. Degree and betweenness hubs were independently defined as nodes in the upper 5% (≥ 95th percentile) of each network's metric distribution. Nodes meeting either criterion were considered network hubs. Associations between mean relative abundance and degree or betweenness were evaluated using Spearman's rank correlations. Differences in degree and betweenness among the four

skin-associated categories were evaluated using Kruskal-Wallis tests followed, when significant, by Dunn's tests with Benjamini-Hochberg correction. Enrichment of degree- and betweenness-defined hubs between core and non-core ASVs was assessed using Fisher's exact tests, with odds ratios as effect sizes.

Network modules were identified using clusterMaker2 v2.3.4 [31] with the GLay algorithm. Module membership was integrated with taxonomic annotation, ecological classification, and hub status. Associations between module membership and degree- or betweenness-hub status were evaluated using Pearson's χ^2 tests with 10,000 Monte Carlo replicates; Cramer's V quantified association strength, and standardized residuals identified modules contributing most strongly to significant associations.

Global network properties (nodes, edges, average number of neighbors, clustering coefficient, density, heterogeneity, centralization, characteristic path length, and connected components) were calculated in Cytoscape for descriptive comparisons among developmental stages. Given the limited number of biological replicates, network analyses were interpreted as exploratory representations of potential microbial community organization rather than direct evidence of ecological interactions.

Ontogenetic network comparison

Independently inferred tadpole, juvenile, and adult networks were compared based on their global topological properties. To examine changes in the relationship between abundance and network position, mean relative abundance was associated separately with node degree and betweenness within each stage using Spearman's rank correlations. The modular distribution of degree and betweenness hubs was evaluated separately for juvenile and adult networks using the contingency-based approach described above.

Persistence analyses

Ontogenetic persistence was operationally defined as repeated detection of an identical standardized 199-bp ASV sequence across independently processed tadpole, juvenile, and adult datasets. This definition indicates sequence-level continuity across developmental stages and does not imply continuous colonization within individual hosts. Representative ASV sequences standardized to 199 bp were matched by exact sequence identity and classified as tadpole-only (T), tadpole and juvenile but not adult (TJ), or detected across all three stages (TJA).

For each matched ASV, mean relative abundance and network properties (degree, normalized degree, betweenness, module membership, and degree- and betweenness-hub status) were retrieved from the corresponding stage-specific network. To test whether persistence across progressively longer developmental intervals was associated with abundance or network position, values were taken from the last stage in which each ASV was detected: tadpole for T, juvenile for TJ, and adult for TJA. Differences

among T, TJ, and TJA groups in mean relative abundance, degree, and betweenness were evaluated using Kruskal-Wallis tests followed by Dunn's tests with Benjamini-Hochberg correction.

For TJA ASVs, topological trajectories were reconstructed by tracking degree- and betweenness-hub status across stages. At each stage, ASVs were classified as degree-hub, betweenness-hub, both, or non-hub using the stage-specific criteria above, allowing assessment of whether persistent lineages retained, lost, or acquired structurally prominent positions during development.

Statistical analyses and robustness assessment

Unless otherwise specified, analyses were performed in R; statistical significance was defined as $P < 0.05$; and Benjamini-Hochberg correction was applied to post hoc comparisons. Non-parametric methods were used for relative abundance, prevalence, and network centrality metrics, and Cramer's V was reported for contingency-table analyses.

Robustness of selected topological patterns was assessed by resampling. For each developmental-stage network, the association between mean relative abundance and betweenness was evaluated using 1,000 bootstrap resamples with replacement. Random subsampling without replacement removed 10%, 20%, or 30% of ASVs, with 1,000 iterations per level and Spearman correlation recalculated for each iteration. For the tadpole network, the difference in median betweenness between core and non-core ASVs was evaluated using the same procedures. Distributions were summarized by mean, standard deviation, median, and 2.5th -97.5th percentiles.

To assess sensitivity of juvenile persistence-associated topological patterns to biological replication, the juvenile network was independently re-inferred five times using random subsets of four of the 10 juvenile samples, with SPIEC-EASI parameters unchanged. For each replicate, degree and betweenness were compared between TJ and TJA ASVs using Kruskal-Wallis tests. One replicate yielded an edge-free network and could not be evaluated; the remaining four were used to assess consistency of persistence-associated patterns.

Large Language Models usage

Large language models (ChatGPT, OpenAI) were used during manuscript preparation to improve text organization, refine scientific writing, condense and reorganize sections, and assist with language editing. All scientific analyses were conducted by the authors, and all scientific interpretations, figures, statistical analyses, and conclusions were reviewed, verified, and approved by the authors. The authors take full responsibility for the final content of the manuscript.

Results

Skin-associated bacterial communities differ from the sampled aquatic microbial pool

Comparison of bacterial occurrence between pre-metamorphic tadpole skin and surrounding water revealed structured partitioning of the aquatic microbial pool. A total of 947 ASVs were classified into five ecological categories: 72 water-only, 198 shared non-core, 140 skin-restricted non-core, 299 shared core, and 238 skin-restricted core ASVs (Fig. 1a). Thus, most tadpole skin ASVs were consistently detected across hosts, either shared with the sampled aquatic environment or apparently restricted to skin. These fractions also showed distinct taxonomic compositions, with shifts in dominant genera across categories (Fig. 1b).

Ecological categories differed strongly in mean relative abundance and skin prevalence (**Online Resource 1a**). Mean relative abundance differed among categories (Kruskal-Wallis $\chi^2 = 406.5$, df = 3, $P < 2.2 \times 10^{-16}$), with all pairwise comparisons significant after Benjamini-Hochberg correction (Dunn's tests, adjusted $P < 0.001$; **Online Resource 1c**). Skin prevalence showed stronger differentiation ($\chi^2 = 686.68$, df = 3, $P < 2.2 \times 10^{-16}$), with all pairwise comparisons significant except between the two core fractions (Dunn's tests; **Online Resource 1d**). Prevalence was strongly positively correlated with mean relative abundance (Spearman's $\rho = 0.800$, $P < 2.2 \times 10^{-16}$; **Online Resource 1e**).

ANCOM-BC2 provided a complementary comparison of abundance between skin and water (**Online Resource 1b**). Among shared ASVs, 72 core and eight non-core ASVs showed significant differential abundance, whereas 154 and 28, respectively, did not. The proportion of differentially abundant ASVs did not differ between core and non-core fractions ($\chi^2 = 0.31$, df = 1, $P = 0.578$; Cramer's V = 0.049). Thus, consistent occurrence across tadpoles was not contingent on detectable differential abundance between skin and water, indicating complementary dimensions of microbial assembly.

Host filtering shapes the topological organization of the tadpole skin microbiota

The tadpole network comprised 875 nodes and 3,181 edges in a single connected component (Fig. 2a; **Table 1**). Mean relative abundance was unrelated to node degree (Spearman's $\rho = -0.017$, $P = 0.619$) but positively associated with betweenness centrality ($\rho = 0.392$, $P < 2.2 \times 10^{-16}$; **Online Resource 2a**), indicating that more abundant ASVs tended to occupy positions linking network regions.

Node degree did not differ among ecological categories (Kruskal-Wallis $\chi^2 = 5.11$, df = 3, $P = 0.164$). However, degree-defined hubs were enriched among core ASVs: 42/537 (7.8%) core versus 6/338 (1.8%) non-core ASVs (Fisher's exact test, odds ratio = 4.69, $P = 5.70 \times 10^{-5}$). Degree hubs were also nonrandomly distributed across ecological categories ($\chi^2 = 23.6$, $P < 0.001$; Cramer's V = 0.164), occurring most frequently among core ASVs shared with the sampled aquatic pool (10.4%; **Online Resource 2c**).

Betweenness increased from shared non-core to skin-restricted non-core ASVs (Kruskal-Wallis χ^2 = 39.69, df = 3, P = 1.24×10^{-8}; **Online Resource 2b**) and was highest in both core fractions (Dunn's tests, BH-adjusted P < 10^{-8}). The two core fractions did not differ (adjusted P = 0.935), whereas betweenness-defined hubs were not enriched among core ASVs (Fisher's exact test, odds ratio = 1.22, P = 0.529).

Together, core membership was associated with increased representation among highly connected nodes and broadly elevated betweenness, despite no general shift in degree and no enrichment of extreme betweenness hubs. Comparison of the complete and core networks further illustrates the contribution of the consistently maintained fraction to network organization (**Online Resource 2d**).

Skin microbial network architecture differs across developmental stages

We next compared the tadpole network with independently inferred juvenile and adult networks from previously published datasets [19]. Network architecture differed markedly among developmental stages (Fig. 2a-c; **Table 1**): tadpoles had 875 nodes and 3,181 edges in one connected component; juveniles had 1,196 nodes and 12,277 edges in one component; and adults had 308 nodes and 711 edges distributed across seven components.

Global topology also varied across stages (**Table 1**). Average connectivity increased from 7.27 neighbors per node in tadpoles to 22.20 in juveniles and declined to 4.33 in adults. Clustering increased from 0.309 to 0.470 and remained high in adults (0.416). Characteristic path length was 6.05 in tadpoles, 4.44 in juveniles, and 10.25 in adults. Density increased from 0.008 to 0.019 before declining to 0.017. Heterogeneity increased from 0.481 to 0.810 and 0.836, while centralization increased from 0.023 in tadpoles to 0.091 in juveniles and remained elevated in adults (0.083). Thus, adult network contraction and fragmentation were not accompanied by uniform reductions in structural organization.

The relationship between abundance and topology also changed across development (**Online Resource 3**). Abundance was unrelated to degree in tadpoles (ρ = -0.017, P = 0.619) but negatively associated with degree in juveniles (ρ = -0.267, P < 2.2×10^{-16}) and adults (ρ = -0.483, P < 2.2×10^{-16}; **Online Resource 3a, e**). In contrast, abundance was positively associated with betweenness at all stages (tadpoles: ρ = 0.392; juveniles: ρ = 0.361; adults: ρ = 0.579; all P < 2.2×10^{-16}; **Online Resources 2a, 3b, 3f**), with the strongest association in adults.

Hub distributions were nonrandom across modules in juveniles (**Online Resource 3c-d**): degree hubs, χ^2 = 232.29, Monte Carlo P < 0.001, Cramer's V = 0.441; betweenness hubs, χ^2 = 17.75, P = 0.0468, Cramer's V = 0.122. Adults showed similar modular structuring (**Online Resource 3g-h**): degree hubs, χ^2 = 66.28, P = 0.0002, Cramer's V = 0.464; betweenness hubs, χ^2 = 41.84, P = 0.0068, Cramer's V = 0.369. Overall, ontogeny was associated with changes in network size, connectivity, abundance-topology relationships, and modular organization.

Sequence-level persistence across ontogeny is associated with microbial abundance and network topology

Sequence-level matching identified 96 tadpole-only ASVs, 58 detected in tadpoles and juveniles but not adults, and 44 detected across all three stages (**Online Resource 4a**). Thus, despite extensive network reorganization, a subset of bacterial lineages remained detectable throughout ontogeny.

Persistence was associated with abundance (Kruskal-Wallis $\chi^2 = 51.55$, df = 2, $P = 6.40 \times 10^{-12}$; **Online Resource 4b**). TJ ASVs were more abundant than tadpole-only ASVs (Dunn's test, BH-adjusted $P = 4.98 \times 10^{-4}$), while TJA ASVs showed the highest abundance, differing from both tadpole-only (adjusted $P = 2.63 \times 10^{-12}$) and TJ ASVs (adjusted $P = 2.70 \times 10^{-4}$).

Sequence-level persistence across developmental stages was also associated with network topology, with contrasting patterns for degree and betweenness. Degree differed among persistence groups (Kruskal-Wallis $\chi^2 = 79.33$, df = 2, $P < 2.2 \times 10^{-16}$; **Online Resource 4c**): TJ ASVs had the highest degree, exceeding tadpole-only (adjusted $P = 2.95 \times 10^{-4}$) and TJA ASVs (adjusted $P = 2.30 \times 10^{-18}$), while TJA ASVs had lower degree than tadpole-only ASVs (adjusted $P = 2.96 \times 10^{-9}$). Betweenness showed the opposite pattern, being highest among TJA ASVs (Kruskal-Wallis $\chi^2 = 29.25$, df = 2, $P = 4.45 \times 10^{-7}$; **Online Resource 4d**); all pairwise comparisons were significant (Dunn's tests, BH-adjusted $P \leq 0.011$). Thus, extended persistence was associated with lower degree but higher betweenness in the primary analysis.

Among the 44 TJA ASVs, persistence did not imply conservation of topological role. Most (31/44; 70.5%) remained non-hubs throughout development, whereas 13 showed stage-specific trajectories. Three were degree hubs in tadpoles but not later, and three acquired degree-hub status only in adults. For betweenness, two ASVs lost hub status after the tadpole stage, two transiently acquired it in juveniles, two acquired it only in adults, and one became a betweenness hub in juveniles and retained this role in adults (Fig. 2d).

Sensitivity analyses supported the betweenness component but not the degree component of the persistence-topology association. Five juvenile networks were re-inferred using random subsets of four of ten samples (**Online Resource 5**). One produced an edge-free network. Among the four evaluable networks, betweenness differed significantly between TJ and TJA ASVs in three replicates ($P = 7.65 \times 10^{-4}$, 0.0177, and 0.0135), but not in the fourth ($P = 0.0604$). TJA ASVs had higher median betweenness in all four replicates. Degree did not differ significantly in any replicate ($P = 0.175–0.319$). Thus, the betweenness association was directionally consistent and statistically recovered in three of four evaluable networks, whereas the degree pattern was not robust.

Together, bacterial lineages persisting across progressively longer developmental intervals showed greater abundance, while those retained through adulthood exhibited comparatively low degree but high betweenness in the primary analysis. Most persistent ASVs remained non-hubs, while others changed topological roles across development. These patterns support a model in which early host-associated

filtering is associated with the establishment of a subset of skin-associated microorganisms, followed by network reorganization while a fraction persists across ontogeny (Fig. 3).

Discussion

Patterns of host-associated filtering in the early skin microbiota

The marked partitioning of bacterial ASVs between tadpole skin and the surrounding aquatic environment indicates that *Rhinella spinulosa* skin microbiota is not simply a reflection of the sampled water-column microbial pool. Community assembly reflects dispersal, selection, diversification, and drift [4–5], with host-associated environments potentially acting as ecological filters that influence which environmentally available microorganisms become consistently associated with the host. Both environmentally shared and skin-restricted ASVs contributed to the prevalence-defined core, consistent with recurrent environmental recruitment, preferential retention in skin, or both. Because only the water-column community was sampled, skin-restricted ASVs cannot be considered absent from other environmental reservoirs.

The strong relationship between prevalence and relative abundance indicates that consistent skin association involves both frequent occurrence and greater representation. Host-associated filtering can operate through host physiology, immunity, and epithelial properties [32–33]. However, prevalence-based host association was not equivalent to differential abundance relative to water, highlighting occurrence, prevalence, and differential abundance as complementary dimensions of host filtering.

The host-associated distribution of ASVs was also reflected in the organization of the tadpole microbial association network. Core ASVs were more frequently represented among degree-defined hubs and showed higher betweenness than non-core fractions, despite no general increase in degree. Highly connected or central microorganisms may contribute disproportionately to community organization [7–8, 34], suggesting that consistently maintained taxa are nonrandomly embedded in the tadpole network. Because network topology represents statistical associations rather than demonstrated ecological interactions, these patterns indicate structural organization rather than direct functional dependence.

Microbial network architecture differs across developmental stages

Amphibian development involves major changes in habitat, physiology, immunity, and skin structure that can alter microbial communities [35]. Our results extend previous evidence of compositional change across metamorphosis by showing marked differences in network architecture. The juvenile network exhibited the greatest connectivity, clustering, heterogeneity, and centralization, whereas the adult network contracted and fragmented. Such differences are consistent with changes in community

composition and environmental conditions across developmental stages [7–10], although developmental and population-level effects cannot be separated here.

The relationship between abundance and topology also changed: abundance was unrelated to degree in tadpoles but negatively associated with degree in juveniles and adults, while its positive association with betweenness persisted across stages. Thus, abundant taxa in later stages tended to have fewer local connections while occupying positions linking different network regions. Non-random hub distributions further indicate redistribution of network roles during development.

Microbial lineages persist while their network context changes

Despite extensive network reorganization, sequence-level matching identified bacterial lineages detectable across developmental stages. Persistence was associated with greater abundance, although the design cannot distinguish within-host persistence from repeated recruitment of sequence-equivalent bacteria. TJA ASVs showed lower degree but higher betweenness in the primary analysis. Sensitivity analyses consistently showed higher median betweenness for TJA than TJ ASVs, with significance recovered in three of four evaluable networks, whereas degree differences were not recovered. Because abundance was also associated with betweenness, this pattern may partly reflect abundance rather than persistence alone.

Most of the 44 persistent ASVs remained non-hubs, while others gained or lost hub status across development. Thus, persistence of bacterial identity did not imply persistence of network role. Bacterial lineages may persist while occupying different positions within stage-specific networks. Whether such topological flexibility contributes to microbiota stability remains unresolved; centrality alone cannot establish ecological function, and experiments are needed to distinguish host selection, microbial traits, and repeated environmental recruitment.

Study limitations

Developmental stage was confounded with geographic origin because stages were sampled from different localities, preventing separation of ontogenetic, population, and environmental effects. Longitudinal or replicated population sampling will be required to resolve these effects.

Environmental characterization comprised only three water-column samples; therefore, absence from sampled water does not imply environmental absence, and skin-restricted categories are relative to this microbial pool.

Small and unequal sample sizes and differences in network size constrain network inference. Networks should therefore be considered exploratory representations of community organization rather than direct ecological interactions, and global metrics should not be interpreted as direct measures of network complexity. Sensitivity analyses provided partial support for the betweenness pattern but not the degree

pattern. Hub trajectories remain descriptive because they were not tested against explicit null models or alternative inference methods. Larger replicated datasets are needed to establish reproducibility.

Persistence was defined by exact identity of a standardized 199-bp 16S rRNA sequence. Identical amplicons may represent different strains, and sampling different individuals prevents distinguishing continuous host persistence from repeated environmental recruitment. Persistence therefore refers to repeated detection of sequence-equivalent lineages rather than continuous colonization.

Taken together, host filtering, network reorganization, and microbial persistence represent complementary dimensions of skin microbiota assembly. Microbial lineages can persist across ontogeny while their topological roles change, indicating that taxonomic continuity can coexist with extensive reorganization of community structure.

Declarations

Funding

This work was supported by the Agencia Nacional de Investigación y Desarrollo (ANID), through the Millennium Institute Center for Genome Regulation (ICN2021_044).

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Study conception and experimental design: CV, FPC, MAM, MLA. Data collection and analysis: CV, FPC. Writing-original draft: CV. Manuscript review and editing: FPC, MAM, MLA. Funding acquisition: MLA. All authors read and approved the final manuscript.

Data availability

The raw sequences generated in this study have been deposited in the NCBI Sequence Read Archive under accession number PRJNA1529848. Previously published sequencing data analyzed in this study are available in the NCBI Sequence Read Archive under accession number PRJNA1003691. All R scripts required to reproduce the analytical pipeline are publicly available in the corresponding GitHub repository (https://github.com/cvaldiviesog/Rhinella-spinulosa-skin-microbiota.git).

Ethics approval

SAG (Servicio Agrícola y Ganadero) provided the collection permits under Resolutions No. 3085/2000, 2105/2004, and 13/2006. The ethical approvals were associated with Certificate No. 25897-FCS-UCH, issued by the Faculty of Sciences, Universidad de Chile.

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Figures

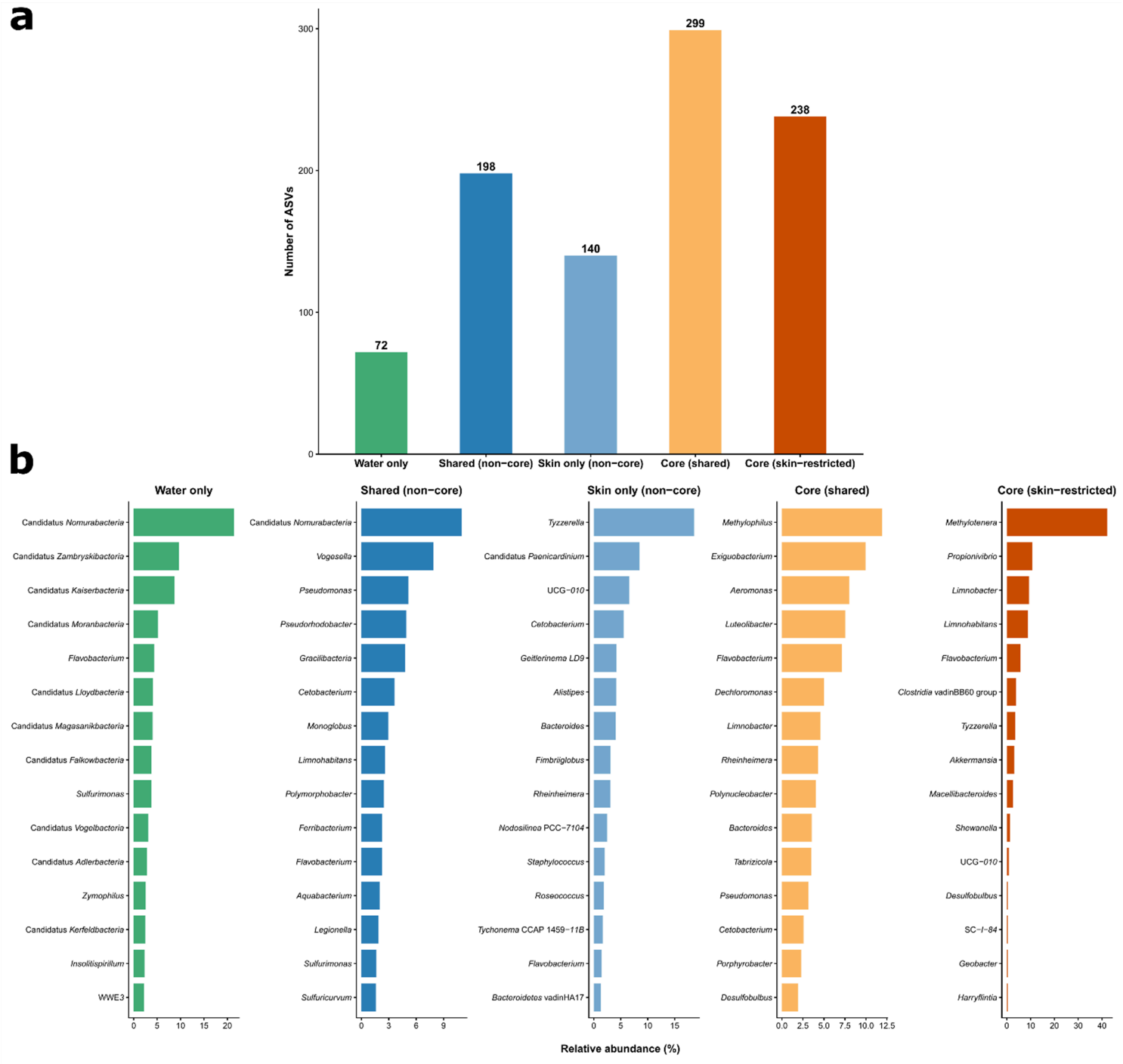


Figure 1

Host-associated partitioning of the skin microbiota relative to the sampled aquatic microbial pool in *Rhinella spinulosa*. (A) Distribution of amplicon sequence variants (ASVs) among five ecological

categories defined according to their occurrence in tadpole skin and environmental water and their prevalence across tadpole individuals. ASVs were classified as water only (detected exclusively in water), shared non-core (detected in both skin and water but present in <80% of tadpoles), skin only non-core (detected exclusively on skin and present in <80% of tadpoles), core shared (detected in both skin and water and present in ≥80% of tadpoles), or core skin-restricted (detected exclusively on skin and present in ≥80% of tadpoles). Numbers above bars indicate the number of ASVs in each category. **(B)**Taxonomic composition of each ecological category at the genus level. Bars represent the mean relative abundance (%) of the most abundant bacterial genera within each ecological category

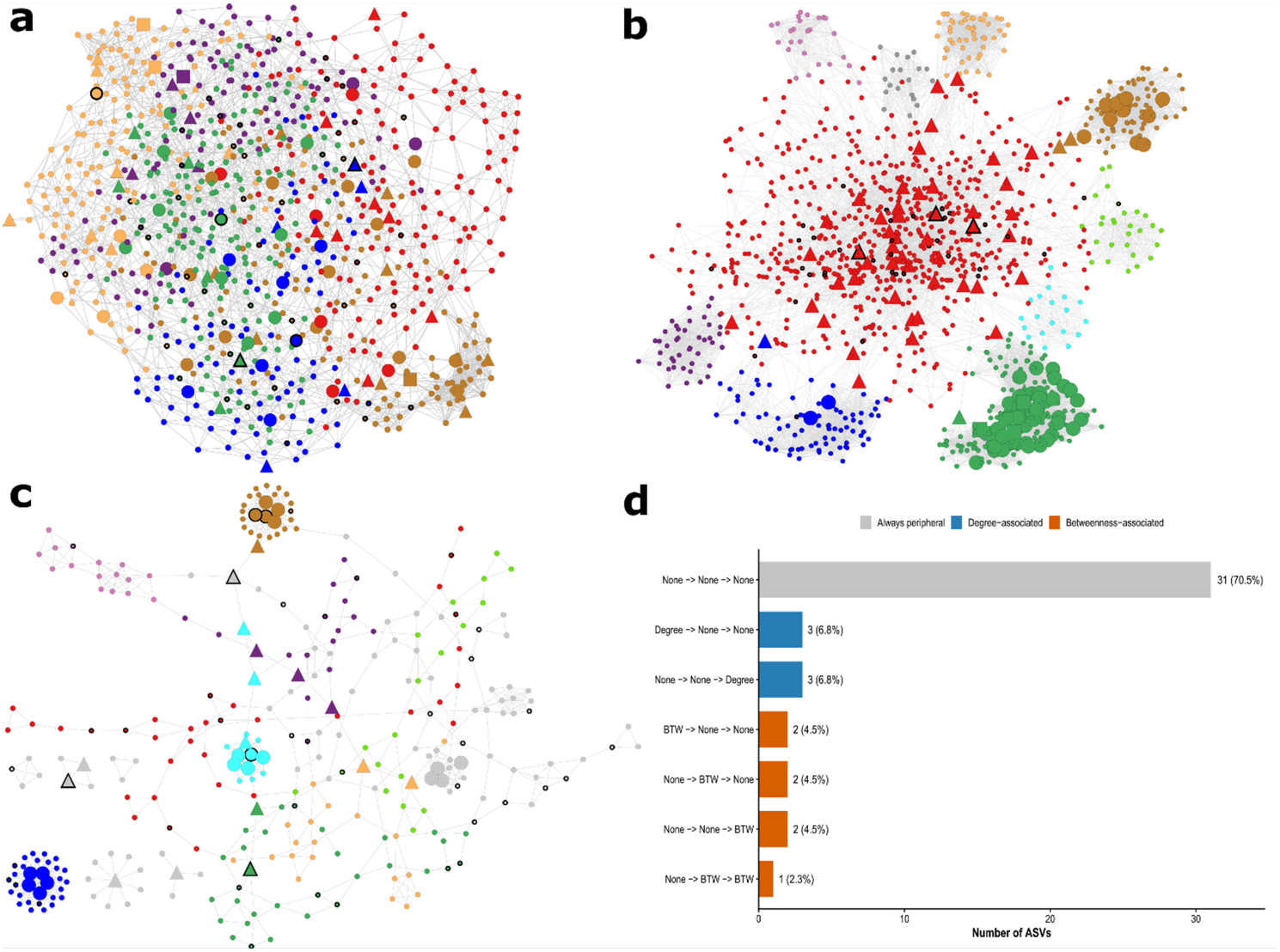


Figure 2

Reorganization of skin microbial association network architecture across developmental stages of *Rhinella spinulosa*. Microbial association networks independently inferred for **(A)** pre-metamorphic tadpoles, **(B)**juveniles, and **(C)** adults. Nodes represent bacterial ASVs, edges represent associations inferred by SPIEC-EASI, and node colors indicate network modules identified using the GLay community detection algorithm. Small nodes represent non-hub ASVs, whereas enlarged nodes indicate network hubs. Enlarged circles represent degree-defined hubs, enlarged triangles represent betweenness-defined

hubs, and enlarged squares represent ASVs classified as both degree and betweenness hubs. Nodes highlighted with a black border correspond to sequence-equivalent ASVs detected across all three developmental stages. **(D)** Topological trajectories of the 44 ASVs detected in tadpole, juvenile, and adult microbiotas, showing changes in hub status across development. Bars indicate the number and percentage of ASVs following each trajectory from tadpoles to juveniles to adults. Gray denotes ASVs that remained non-hubs throughout development, blue denotes trajectories involving degree-hub status, and orange denotes trajectories involving betweenness-hub status. “None” indicates that an ASV was present but was not classified as a hub at the corresponding developmental stage

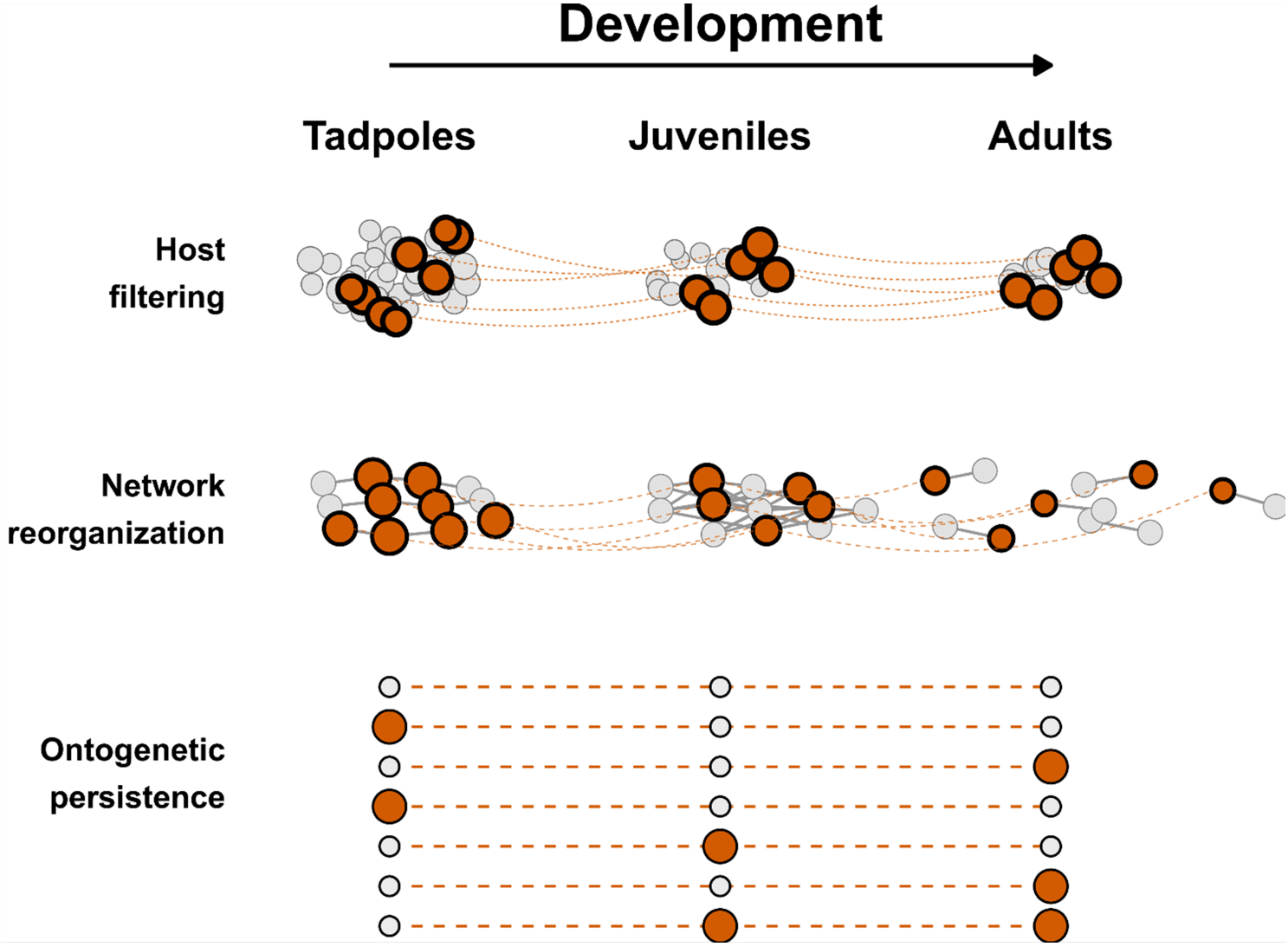


Figure 3

Conceptual model integrating host filtering, microbial network reorganization, and bacterial lineage persistence across development in *Rhinella spinulosa*. During the pre-metamorphic tadpole stage, host filtering structures the skin microbiota by selecting a subset of bacteria from the surrounding environmental microbial pool, including both environmentally shared and skin-restricted taxa. Across subsequent developmental stages, microbial association networks undergo extensive reorganization, with changes in network architecture and in the topological positions occupied by individual bacterial

taxa. Despite this reorganization, a subset of bacterial lineages remains detectable across successive developmental stages, providing a component of taxonomic continuity throughout ontogeny. Orange nodes represent bacterial taxa retained by the host-associated microbiota, and gray nodes represent other members of the environmental or stage-specific microbial pool. Dashed orange lines connect persistent bacterial lineages across stages, illustrating ontogenetic persistence rather than direct ecological interactions. Together, the model emphasizes that bacterial lineage persistence can coexist with extensive reorganization of microbial community structure and does not require conservation of network topology across host development

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.xlsx
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- OnlineResource4.pdf
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